Implant Dentistry

Effect of titanium surface on secretion of IL1β and TGFβ1 by mononuclear cells

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Abstract: Mononuclear cells play an important role in the modulation of healing. The characteristics of implant surface topography may alter the production of signaling molecules such as cytokines. The aim of this *in vitro* study was to evaluate the effects of commercially available titanium surface treatments on both cell viability and the secretion of the antagonist cytokines, IL1β and TGFβ1. Human mononuclear cells were cultured on 10 mm diameter commercially pure titanium (cpTi) disks that were prepared using a turning procedure (control=machined surface) and either acid etched or bio-anodized for 1-7 days. Adhered cells were investigated with respect to cell viability using an MTT assay, and cytokine production was verified using an ELISA assay. The results indicate that surface characteristics did not alter the cell viability at days 1 and 4, although the machined surface presented the highest absorbance values at day 7 ($p = 0.0084$). Cell viability was reduced throughout the time course for all analyzed surfaces ($p < 0.05$). On day 4, IL1β levels were significantly higher on bio-anodized compared to acid etched surfaces ($p = 0.0097$). TGFβ1 did not show differences among the surfaces at days 1 and 4. The responses of non-stimulated mononuclear cells to titanium surfaces suggest only modest effects of the surface treatment and roughness on pro-inflammatory cytokine (IL1β) release.

Descriptors: *In vitro*; Interleukin-1; Transforming Growth Factor Beta; Titanium.

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Introduction

The placement of endosseous implant materials evokes a generalized wound healing response that involves vascular, humoral and cellular aspects of inflammation.[1,2] Mononuclear cells show considerable importance in initial peri-implant healing,[1-3] as they are involved in chemokines,[1,4-5] cytokines[5,6] and growth factors releasing[7] into the extracellular environment.

The mediators released by mononuclear cells can be divided into two main categories: pro-inflammatory, such as IL1β,[1-2,4-5] TNFα,[1,4-6] IL6,[1-2,4-5] or anti-inflammatory or modulatory, such as IL10[4-6,8-9] and TGFβ.[10] The cytokine profile released by monocytes/macrophages around dental implants is modulated by the surface characteristics of titanium and titanium alloys used to produce the implants.[1,6] Studies conducted with mononuclear cells have demonstrated substrate-dependent effects in cell activation.[1,5,8-9,11] Once a biomaterial surface able to accelerate the heal-

ing time is desirable, modifications on implant shape and surface have been done to obtain better biological responses, minimizing the mineralization time.[12]

A wide variety of titanium surface treatments that are currently proposed by the scientific community result in different surface compositions and energy and topographical conditions.[12-14] However, there is no consensus regarding the best surface treatment for enhancing the biological response and achieving superior bone healing.[15] Techniques able to produce microrough surfaces, such as sandblasting, acid etching and electrochemical anodization, continue to be used by the implant industry to manufacture dental implant surfaces.[12,15-16] The methods used to obtain acid-etched surfaces, for instance, show little differences regarding the acid concentration, time of exposition and type of acid used.[16,17] It is thus important that these commercially available surfaces continue to be evaluated to determine the effects of the surface characteristics of certain implant biomaterials on cell behavior and, furthermore, on bone healing. Thus, the aim of this study was to analyze the effects of three titanium surface treatments, machined, anodized or acid etched, on cell viability and on the release of the antagonist cytokines IL1β and TGFβ1 by human mononuclear cells. The null hypothesis is that any titanium surface treatments increase cell viability and the release of antagonist cytokines.

Methodology

Commercially pure titanium (cpTi grade 4) disks (10 mm diameter and 4 mm thickness) were manufactured by Conexão Sistema de Próteses (São Paulo, Brazil), with three types of surface treatments:

- machined,
- acid etched and
- anodized.

The machined disks were obtained from cpTi bars in a turning procedure and did not receive any additional treatment and were used as control. The acid-etched titanium discs were obtained by immersion in a mixture of HNO_3, HCl and H_2SO_4, resulting in surfaces with a surface roughness mean (Ra) of approximately 0.51 µm. The anodized samples were prepared using micro-arc oxidation with electrolyte solution containing calcium and phosphorus at high anodic forming voltages and a galvanostatic current[16] showing surface roughness mean (Ra) of approximately 0.87 µm. The roughness and hydrophobicity of the referred implant surfaces were previously evaluated using a scanning electron microscopy, profilometry,[12,17] thin-film X-ray diffraction and X-ray photoelectron spectroscopy.[16]

Ethical considerations

This research project was approved by the Ethics Committee of the Federal University of Uberlândia, MG, Brazil, and written informed consent was obtained from all blood donors.

Cell culture

Whole blood from ten healthy adult volunteers was drawn into heparinized tubes and diluted at 2:1 in Hanks balanced saline solution (HBSS) (pH 7.2-7.4). From each donor, diluted blood (7 ml) was carefully layered over 3 ml of gradient separation medium (Percoll™, St. Louis, USA) in a conical centrifugation tube and centrifuged at room temperature for 30 min at 800 g to separate monocytes and lymphocytes from remaining blood elements. The interface, containing mononuclear cells, was aspirated and transferred to a sterile tube, washed with HBSS and centrifuged at 1000 g for 10 min at 4 °C. The mononuclear cells were stained with trypan blue, counted and resuspended in RPMI 1640 (Sigma Chemical Company, St. Louis, USA) containing 5% fetal calf serum (FCS) and 1% penicillin-streptomycin (Gibco-Life Technologies, Grand Island, USA). From each patient, 1 ml of the filtered blood plasma fraction was used to incubate disks for 10 min and was then aspirated away. Mononuclear cells were then plated onto Ti surfaces at 1×10^6 cell/ml/well and were incubated for 3 hours in a humidified, 5% CO_2 incubator at 37 °C to allow the cells to settle on the different surfaces. Then, the plates were centrifuged at 400 g for 10 min, and the non-adherent cells were removed by aspiration. Fresh medium was added to each well, and the plates were then returned to the incubator and cultivated over the course of 7 days. The samples were analyzed at

days 1, 4 and 7. For each donor, in each period, the disks were analyzed in triplicate.

Cell viability

Cell viability was estimated at days 1, 4 and 7 using a mitochondrial colorimetric assay (MTT assay). Briefly, in each well, 600 µl of culture medium was aspirated, leaving 400 µl in which 40 µl of MTT solution (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (Sigma Chemical Company, St. Louis, USA) was added. Cells were then incubated at 37°C and 5% CO_2 for 4 h. The upper medium was removed carefully, and the intracellular formazan was solubilized by adding 400 µl of dimethylsulfoxide (Sigma Chemical Company, St Louis, USA) to each well. Then, the contents of the wells were mixed thoroughly using a pipette, and 200 µl of each well was transferred to a separate well on a 96-well ELISA plate (Corning Costar, Lowell, USA). The absorbance was measured at a wavelength of 570 nm. The results, which were expressed in optical density (OD), were obtained for three different experiments from each surface modification.

Cytokine levels

IL1β and TGFβ1 levels were measured at days 1 and 4 using sandwich enzyme-linked immunosorbent assays (ELISAs) in accordance with the manufacturer's protocols (e-Bioscience San Diego, USA). The optical density of each well was determined using a microplate reader set to 450 nm and corrected at 570 nm. The samples values were determined by comparison with standard curve. All samples were assayed in triplicate.

Statistical analysis

Data were normality evaluated using the D'Agostino & Pearson omnibus normality test. One-way ANOVA and then Tukey's test were used for cell viability. A Kruskal-Wallis test followed by Dunn's Multiple Comparison Test was performed for cytokine measurements at days 1, 4 and 7. Values of $p < 0.05$ were considered significant.

Results

Cell viability

Figure 1 shows the results of the viability assay, expressed as absorbance levels. On day 1 (Figure 1A), no significant differences were observed between titanium roughness surfaces and the control surface ($p = 0.0921$). Similarly, on day 4, mononuclear cell viability was significantly similar when cells were cultivated on cpTi disks, independent of the surface characteristic ($p = 0.6794$; Figure 1B). However, cell viability presented a significant statistical difference on day 7 when comparing control and machined groups ($p = 0.0084$; Figure 1C). During this period, the control surface demonstrated significantly higher absorbance values in the MTT assay when compared to the acid-etched or anodized groups (Figure 1C). All groups showed significant reduction in cell viability from days 1 to 7 ($p < 0.05$).

Cytokine levels

Contact with different titanium surfaces caused

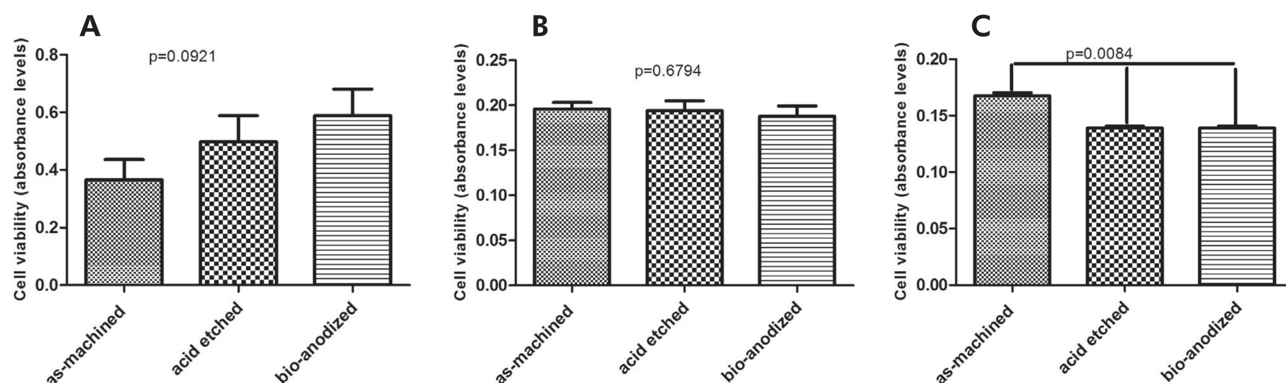

Figure 1 - Cell viability at day 1 **(A)**, 4 **(B)** and 7 **(C)**. Data expressed as absorbance levels ($p < 0.05$).

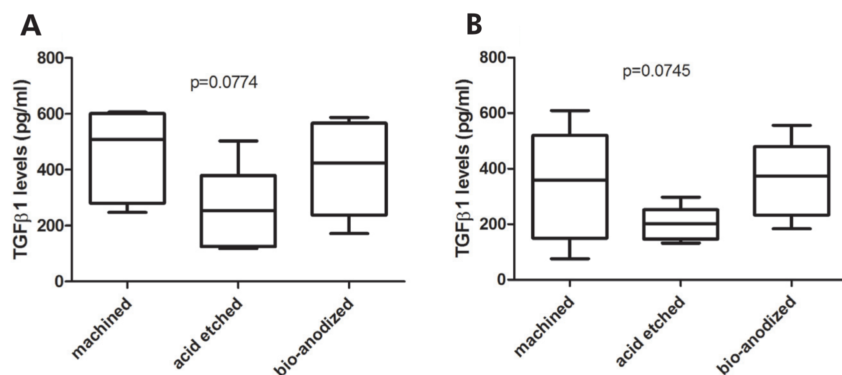

Figure 2 - TGFβ1 levels secreted by mononuclear cells cultured on machined, acid etched and bio-anodized surfaces at day 1 **(A)** and 4 **(B)** ($p < 0.0$).

the release of IL1β and TGFβ1 from human mononuclear cells. On day 1, IL1β release by mononuclear cells adhered on rough surfaces (acid etched or anodized groups) did not show significant statistical differences when compared to cells cultured on the control surface ($p = 0.1006$). However, at day 4, the cells cultured on the anodized surface released significantly higher IL1β levels than cells on the control surface ($p = 0.0097$). Regarding cytokine levels throughout the time course, the acid etched surface was the sole group in which a statistically significant effect on IL1β levels was observed ($p = 0.0152$). No significant differences were found on TGFβ1 levels detected in media from cultures grown on modified titanium surfaces (etched acid or anodized groups) compared to the control surface (day 1: $p = 0.0774$; day 4: $p = 0.0745$; Figure 2). Additionally, cells grown on the three Ti surfaces did not show significant differences in TGFβ1 levels throughout the time course ($p > 0.05$).

Discussion

The null hypothesis was partially rejected. An increase in cell viability was not observed in any group; however, IL1β secretion was higher on the anodized surface on day 4 compared to the other groups.

The present *in vitro* study investigated the influence of two commercially available treated dental implant surfaces, produced by means of acid etch or anodization, on the response of human mononuclear cells, mainly monocytes/macrophages. Though the effects of nano- and microrough titanium topography on osteoblast behavior have been extensively described,[18-19] assays in which monocyte/macrophage behavior is examined are useful determinants for elucidating several aspects of cell interactions with implant biomaterials.[6,11,20]

Mononuclear cells, especially monocytes/macrophages, were chosen for this research due to their pivotal role in wound healing,[8] producing a range of molecules which have the potential to modulate the repair process, driving the duration and intensity of the inflammatory response.[20] According to this perspective, it is reasonable to hypothesize that the interaction between mononuclear cells and the implant surface influences the subsequent phases of *de novo* bone formation.[1,21] This assumption stimulated the current research, in which we used single cultures of human mononuclear cells as a biological model.

Previous studies suggest that both the methodology and the cells used to evaluate the interaction between macrophage/implant may influence the results.[2,20] Although human[5] and murine macrophage cell lines[1,2,7,22] have been often used in previous models to study the interaction between biomaterial surfaces and monocytes/macrophages, human primary cells obtained from peripheral blood are closer to actual clinical conditions. In the present study, the cells obtained from blood samples were subjected to an additional centrifugation step after cell plating to remove lymphocytes, increasing the relative number of monocytes.[10] This methodology results in more than a 90% macrophage among the adherent cells.[10,21] In the current study, it was desirable to obtain the highest possible purity of monocytes, reducing the influence of other cells on the monocyte/macrophage behavior after contact with the titanium surfaces.

The treatment used to obtain rough surfaces did not influence cell viability until day 4. Previous

studies have demonstrated that macrophages prefer rough surfaces to smooth ones.[1] Other factors such as protein adsorption may also influence cell behavior. However, the highest absorbance levels observed on the machined group on day 7 represents an intriguing result. This finding strongly suggests that physiological cell death was reduced in the machined group. It is possible that the immersion in plasma proteins before cell seeding may have modified the interaction between monocytes/macrophages and the titanium surfaces. Furthermore, it is important to address that the decision to use the turned titanium surface as the control group in accordance with Göransson *et al.*[11] According to Gretzer *et al.*,[8] macrophages cultured on smooth titanium surfaces did not show differences regarding this parameter when compared to a polystyrene surface.

The reduction observed in cell viability throughout the time course for all groups is explained by the intrinsic characteristic of these cell cultures. Taking into account the fact that primary and non-stimulated cell cultures were used and that monocytes are comprehensively differentiated cells, these results were predictable. These findings are in agreement with a prior study,[11] which observed a decrease in the total number of viable cells on all titanium surfaces over time.

Considering that the cytokine profile at the peri-implant site is related to wound healing and bone formation, the secretion of two antagonist cytokines, IL1β and TGFβ1, were analyzed. These cytokines were previously evaluated by others,[4,20] and they are considered classic for studies seeking to understand the transition between inflammation to wound healing.

IL1β is considered a key mediator of the inflammatory process, which also contributes to the reparative phase, inducing the releasing of additional cytokines and growth factors.[1,20] In the present study, the low levels of IL1β are in agreement with previous studies,[5,20] hich observed little response of unstimulated macrophages cultured over rough surfaces. However, the slight difference observed in the anodized group on day 4 when compared to the acid-etched group could be related to calcium and phosphorus deposition on the titanium surface. These ions may be acting as antigenic materials, stimulating the release of cytokines with pro-inflammatory behavior. The pattern of secretion of IL1β on days 1 and 4 showed increased levels only for cells grown on the acid-etched surface. Cytokine quantification was not performed on day 7 due to considerable reduction on cell viability in all groups.

The other mediator evaluated, TGFβ1, is one of the most important growth factors of human bone, stimulating proliferation and differentiation of pre-osteoblasts,[23]and modulating wound healing, promoting angiogenesis and collagen synthesis.[24] TGFβ1 secretion was detected from cells on all analyzed surfaces, without any statistically significant differences. The similar level of secretion of TGFβ1 in experimental and control groups reinforces the inability of these modified surfaces to stimulate an increase of growth factor secretion, such as TGFβ1.

Taking into account that the present study sought to mimic early events after implantation, imitating the interaction between the cell, plasma proteins and surfaces, the lack of differences on the analyzed parameters may indicate the influence of adsorbed proteins on cell behavior. Within the proposed methodology, the present study suggests that the treatment used to obtain the rough titanium surfaces (acid etched or anodized), as well as the differences in roughness described by both surfaces,[12,16] did not influence the pro-inflammatory or anti-inflammatory cytokine profile, using as a model IL1β and TGFβ1 release by human mononuclear cells. Furthermore, it is important to recognize that the current study evaluated cpTi surfaces that had been pre-coated with plasma proteins, which can alter the interaction with the cell surface and the secretion of cytokines from the cell. These results indicate that mononuclear cells are inert to these surfaces, which were previously coated with blood plasma.

Conclusion

Notably, the commercially available surfaces evaluated in the present study, which were pre-coated with plasma proteins, did not possess an influence on mononuclear cell viability or activation as demonstrated by absorbance levels and IL1β and TGFβ1 analysis.

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